BATF is required for normal expression of gut-homing receptors by T helper cells in response to retinoic acid

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CCR9 and α4β7 are the major trafficking receptors for lymphocyte migration to the gut, and their expression is induced during lymphocyte activation under the influence of retinoic acid (RA). We report here that BATF (basic leucine zipper transcription factor, ATF-like), an AP-1 protein family factor, is required for optimal expression of CCR9 and α4β7 by T helper cells. BATF-deficient (knockout [KO]) mice had reduced numbers of effector T and regulatory T cells in the intestine. The intestinal T cells in BATF KO mice expressed CCR9 and α4β7 at abnormally low levels compared with their wild-type (WT) counterparts, and BATF KO CD4$^+$ T cells failed to up-regulate the expression of CCR9 and α4β7 to WT levels in response to RA. Defective binding of RARα and histone acetylation at the regulatory regions of the *CCR9* and *Itg-α4* genes were observed in BATF KO T cells. As a result, BATF KO effector and FoxP3$^+$ T cells failed to populate the intestine, and neither population functioned normally in the induction and regulation of colitis. Our results establish BATF as a cellular factor required for normal expression of CCR9 and α4β7 and for the homeostasis and effector functions of T cell populations in the intestine.

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Abbreviations used: BATF, basic leucine zipper transcription factor, ATF-like; ChIP, chromatin immunoprecipitation; HDAC, histone deacetylase; iT_{reg} cell, induced T regulatory cell; LP, lamina propria; MLN, mesenteric LN; PLN, peripheral LN; PP, Peyer's patch; RA, retinoic acid.

Effective immunity and immune tolerance require optimal migration and population of lymphocytes in various tissues in the body (Williams, 2004; Kim, 2005; Ley et al., 2007). Tissue-specific migration of lymphocytes is possible through distinct expression of trafficking receptors by lymphocyte subsets. Gut-homing lymphocytes preferentially express a chemokine receptor, CCR9, and an integrin, α4β7 (Hamann et al., 1994; Berlin et al., 1995; Abitorabi et al., 1996; Mackay et al., 1996; Zabel et al., 1999; Kunkel et al., 2000; Papadakis et al., 2000; Wurbel et al., 2000; Marsal et al., 2002; Svensson et al., 2002; Pabst et al., 2004). In contrast, skin-homing T cells express other trafficking receptors such as cutaneous lymphocyte-associated antigen, CCR4, CCR8, and/or CCR10 (Sigmundsdottir and Butcher, 2008).

CCL25, a chemokine expressed by epithelial cells in the small intestine, activates CCR9 for adhesion triggering and chemotaxis (Vicari et al., 1997; Zabel et al., 1999; Kunkel et al., 2000; Wurbel et al., 2000). α4β7 is expressed by T and B cells that migrate to the Peyer's patches (PPs) and lamina propria (LP) of the small intestine and colon (Holzmann and Weissman, 1989; Erle et al., 1994; Hamann et al., 1994). Both CCR9 and α4β7 are induced by retinoic acid (RA), a nuclear hormone produced in the gut by retinaldehyde dehydrogenase (RALDH)–expressing dendritic cells and epithelial cells (Niederreither et al., 2002; Iwata et al., 2004). It has been determined that expression of the α4 chain of α4β7 is induced by RA (Kang et al., 2011). Integrin β7 is constitutively expressed but can be further up-regulated by TGFβ1 and RA (Kilshaw and Murant, 1991; Kang et al., 2011). RARα would work together with other transcription factors such as NFATc2 to induce the expression of CCR9 by T cells (Ohoka et al., 2011). These RA-induced trafficking receptors regulate migration of IgA-producing B cells and effector T cells (Iwata et al., 2004; Mora and von Andrian, 2009; Wang et al., 2010).

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BATF (basic leucine zipper transcription factor, ATF-like) is a basic leucine zipper (b-Zip) transcription factor of the AP-1 protein family (Dorsey et al., 1995). BATF is widely expressed in the immune system, including T and B cells. It heterodimerizes with Jun proteins for transcriptional regulatory activity (Dorsey et al., 1995; Echlin et al., 2000; Williams et al., 2001). BATF is required for the generation of Th17 cells and T-Fh cells but is dispensable for development of Th1 cells and FoxP3$^+$ T cells (Schraml et al., 2009; Betz et al., 2010; Ise et al., 2011). It has been reported that BATF can suppress *Sirt1* expression and control the ATP level and effector function of CD8$^+$ T cells (Kuroda et al., 2011). Additionally, BATF deficiency is associated with the loss of activation-induced cytidine deaminase (AID) expression and class switch recombination in B cells (Betz et al., 2010; Ise et al., 2011), and BATF recently has been shown to regulate a DNA damage–induced differentiation checkpoint important for the maintenance of hematopoietic stem cells (Wang et al., 2012).

We report here that BATF is required for optimal expression of CCR9 and α4β7 by gut-homing CD4$^+$ T cells in response to the RA signal. BATF KO mice are numerically deficient for T cells in the intestine. BATF-deficient effector T helper cells and FoxP3$^+$ T cells are ineffective in migration into the intestine and fail to function as effector cells and suppressor cells, respectively. BATF is required for CD4$^+$ T cells to up-regulate the gut-homing receptors in response to RA upon antigen priming and to migrate into and populate the intestine.

RESULTS

T helper cells are numerically deficient in the intestine of BATF KO mice

BATF KO mice generated by targeted deletion of either exons one and two or exon three of the *Batf* gene have been previously described to have relatively normal numbers of T cells in secondary lymphoid tissues (Schraml et al., 2009; Betz et al., 2010). When we examined the intestine by immunohistochemistry, CD4$^+$ T cells were numerically deficient in the LP compartment of the small intestinal villi (Fig. 1 A). CD8$^+$ T cells were present but also decreased in numbers in the small intestinal villi of the BATF KO mice. Flow cytometry analysis revealed that the frequency of CD4$^+$ T cells was decreased significantly in the colon and the small intestinal LP (Fig. 1 B). CD4$^+$ T cells were decreased also in PPs but not in the mesenteric LN (MLN). When FoxP3$^+$ CD4$^+$ T cells were examined, they were more decreased in the colon and the small intestine than in MLN or PPs (Fig. 1, B and C). CD8$^+$ T cells were significantly decreased in PPs and the small intestine (Fig. 1, A–C).

In an effort to provide an explanation for the deficiency of CD4$^+$ T cells in the intestine, we examined the expression of CCR9 and α4β7 (Fig. 2 A). Both FoxP3$^+$ and FoxP3$^-$ CD4$^+$ T cells in the small intestine of BATF KO mice expressed CCR9 at reduced levels compared with WT mice (Fig. 2, A and B). Numbers of CD4$^+$ FoxP3$^+$ T cells positive for α4β7 were reduced in the small intestinal LP of BATF KO mice (Fig. 2, A and B). A considerable decrease in CCR9 and α4β7 expression was also detected in CD8$^+$ T cells in the small intestine but not in other organs (Fig. 2 C).

Naive BATF KO CD4$^+$ T cells are ineffective in up-regulating CCR9 and α4β7 in response to RA

Expression of CCR9 and α4β7 by T cells is induced by RA (Iwata et al., 2004). We next examined whether BATF KO naive T cells are defective in up-regulating trafficking receptors in response to T cell activation in the presence of RA in vitro. Compared with WT CD4$^+$ T cells, BATF KO naive T cells showed less up-regulation of CCR9 in response to RA (Fig. 3 A). Similarly, BATF KO naive T cells were unable to up-regulate α4β7 to WT levels. Similar results were obtained with concanavalin A (Fig. 3 A) or with $OVA_{323–339}$ when OT-II BATF KO T cells were used (not depicted). At the mRNA level, expression of both *CCR9* and *Itg-α4* in response to RA was defective in BATF KO T cells (Fig. 3 B). However, expression of *Itg-β7* mRNA by BATF KO T cells was comparable with WT T cells. We examined other chemokine receptors such as CCR4, CCR6, CCR7, and CXCR4 (Fig. 3 C) and did not detect any significant differences in expression of these receptors between WT and BATF KO T cells cultured with or without RA. RA enhances the induction of FoxP3$^+$ T cells by TGFβ1 (Kang et al., 2007; Mucida et al., 2007), and this response was intact in BATF KO T cells (Fig. 3 D). However, BATF KO T regulatory cells (iT_{reg} cells) induced with RA and TGFβ1 were deficient in expression of CCR9 and α4β7 (Fig. 3 E). These results indicate that expression of gut-homing receptors in BATF KO T helper cells to RA is defective, whereas differentiation of T_{reg} cells was normal.

BATF KO T cells regain the ability to express α4β7 and CCR9 upon gene complementation

It is possible that BATF KO T cells are defective in expression of CCR9 and α4β7 as the result of a block in cell development rather than the absence of functional BATF. This was addressed using retroviral gene complementation in BATF KO T cells. We constructed retroviral vectors expressing the full-length and two truncated versions of BATF and used these to complement the BATF deficiency in T cells (Fig. 4 A). Enforced BATF expression induced a low level of α4β7 but did not induce CCR9 in the absence of exogenous RA (Fig. 4, B and C). This modest induction of α4β7 is likely caused by residual RA that is present in the culture medium. BATF KO CD4$^+$ T cells were able to normally express CCR9 and α4β7 in response to RA when the full-length BATF protein was expressed (Fig. 4, B and C). A low but detectable activity was observed for a BATF mutant deleted for the first exon (BATFΔ2), and this was predicted because the protein retains the Jun dimerization and DNA-binding domains of BATF (Fig. 4, B and C). In contrast, a nonfunctional BATF protein deleted for the first and second exons (BATFΔ1) did not have such activities.

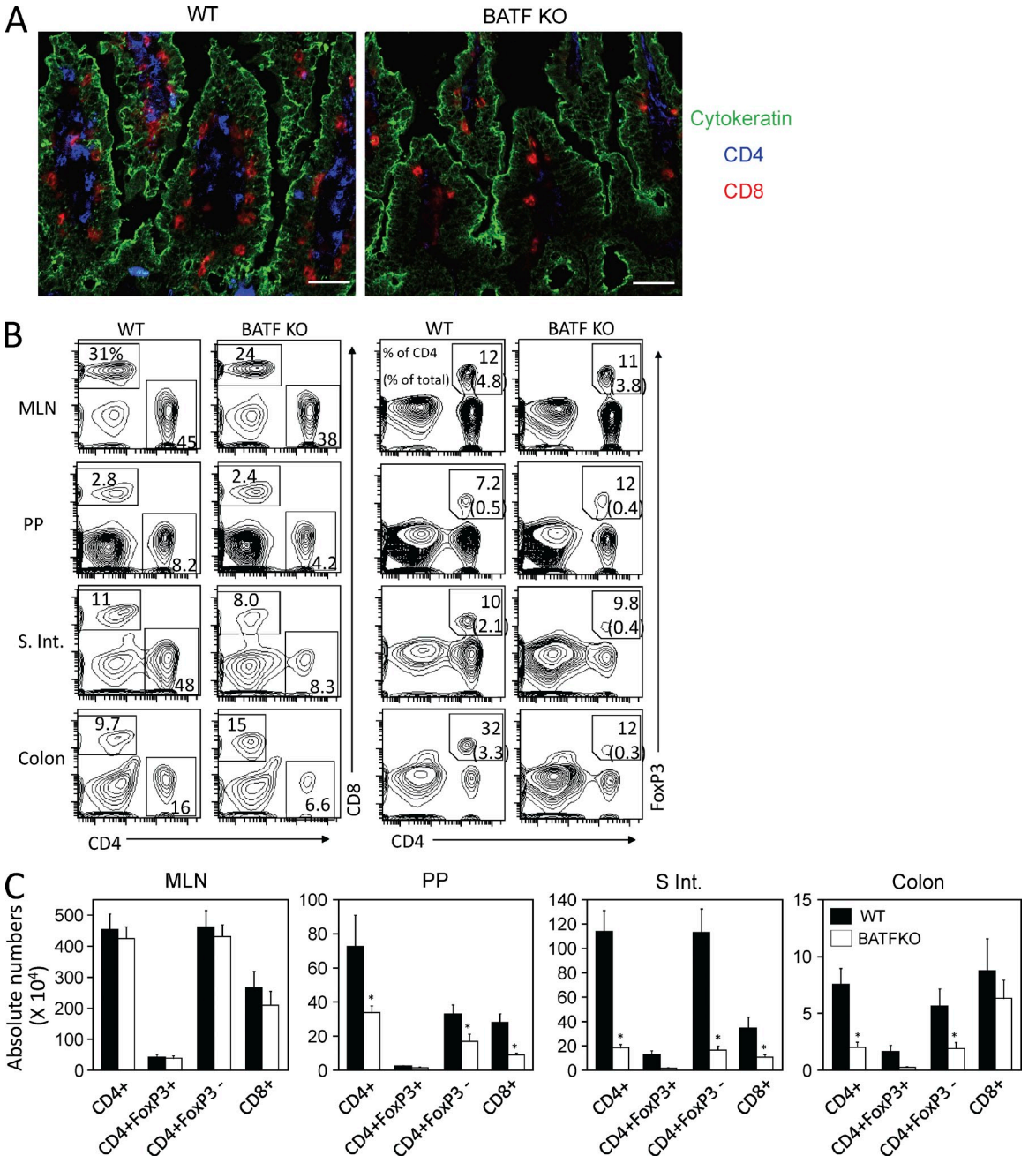

Figure 1. BATF KO mice are deficient with T cells in the intestine. (A) CD4 and CD8 cells in the small intestinal villi of WT and BATF KO mice were examined by immunohistochemistry. Bars, 50 µm. (B) $CD4^+$ $FoxP3^-$ effector, $CD4^+$ $FoxP3^+$ regulatory, and $CD8^+$ T cells in selected organs were analyzed by flow cytometry. (C) Absolute numbers of each cell population in the indicated organs. Representative (A and B) and pooled (C) data obtained from four experiments using 6–8-wk-old mice are shown. All error bars are SEM obtained from pooled data. Significant differences from WT T cells are shown (*, $P < 0.05$).

These results demonstrate that BATF KO $CD4^+$ T cells are not developmentally defective but rather are functionally defective in expression of the gut-homing receptors.

Defective binding of RARα to the regulatory regions of the *CCR9* and *Itg-α4* genes in BATF deficiency

To gain insights into the defective expression of CCR9 and α4β7 in BATF deficiency, the expression of RARα, RARβ, and RARγ was examined and was found to be normal in BATF KO T cells cultured in the presence and absence of RA (Fig. 5 A). We examined the sequence structure of the regulatory region of the *CCR9* and *Itgα4* genes. We found several RARα and BATF binding sites in the 5′ upstream regions of the mouse *CCR9* and *Itgα4* genes (Fig. S1 and Table S1). Chromatin immunoprecipitation (ChIP) followed by genomic DNA PCR on WT T cells activated in the

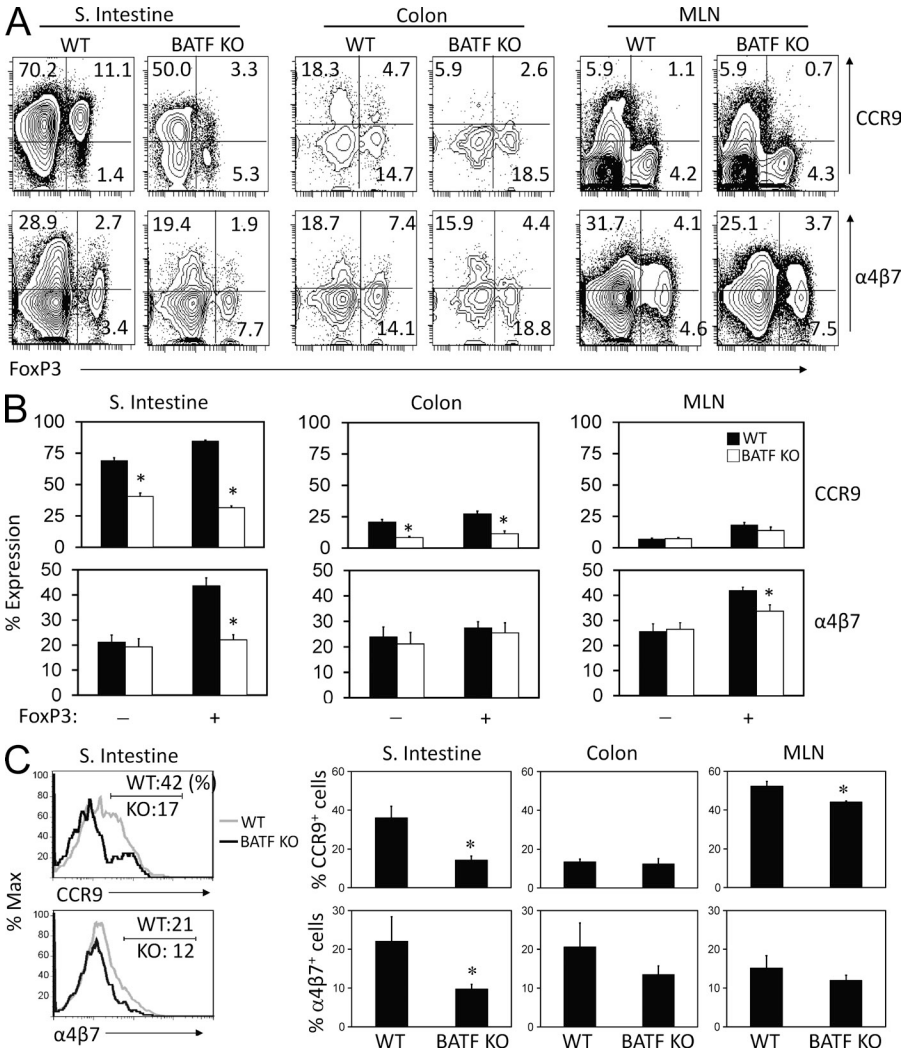

Figure 2. BATF KO gut T cells are deficient in expression of CCR9 and α4β7. (A) Expression of the indicated trafficking receptors by CD4+ FoxP3+/− T cell subsets were analyzed by flow cytometry. (B) Graphs show absolute cell numbers. (C) Expression of CCR9 and α4β7 by CD8+ T cells. Graphs show frequencies of CCR9+ or α4β7+ CD8+ T cells in the indicated organs. Representative (A) and pooled (n = 8–11 for B; n = 5 for C) data obtained from at least five experiments using 6–8-wk-old mice are shown. All error bars are SEM obtained from pooled data. Significant differences between WT and BATF KO T cells are shown (*, $P < 0.05$).

presence of RA revealed the binding of RARα and BATF to 18 regions in the 44-kbp 5′ upstream region of the *CCR9* gene as well as to the 5′ upstream region of the *Itgα4* gene (Fig. 5, B and C). The binding of RARα to the *CCR9* and *Itgα4* genes was abnormally low in BATF KO T cells (Fig. 5, B and C). Moreover, the level of acetylated histone 4 protein was very low in the regulatory regions of *CCR9* and *Itgα4* genes. In line with this, the promoter activity of a 1.3-kbp region spanning the 5′ upstream regulatory region of *Itg-α4* gene was significantly lower in BATF KO than WT T cells (Fig. 5 D).

Nuclear hormone receptor function involves a series of enzymatic modifications of proteins including the acetylation of histone proteins for gene expression (Rosenfeld et al., 2006). It has been reported that expression of Sirt1, a histone deacetylase (HDAC) class III enzyme, is induced in BATF deficiency to decrease acetylation of histones in CD8+ T cells (Kuroda et al., 2011). To determine the role of histone acetylation in expression of CCR9 and α4β7 in BATF deficiency, we used inhibitors of HDACs (trichostatin A and BML-210 for class I/II; Ex-527 for Sirt1). Increased acetylation through class I and II deacetylase inhibitors restored the expression of the gut-homing receptors (Fig. 5 E). However, inhibition of Sirt1 was not effective.

BATF KO T cells fail to migrate to various compartments of the intestine

Defective expression of CCR9 and α4β7 is expected to negatively influence the homing of BATF KO T cells to the gut. We determined the short-term homing ability of BATF KO T cells in vivo. Naive CD4+ T cells from WT and BATF KO mice were activated in the presence of RA for 6 d and examined for chemotaxis to the CCR9 ligand CCL25 in vitro (Fig. 6 A). Chemotaxis by RA-treated BATF KO CD4+ T cells was inefficient compared with WT T cells (Fig. 6 A), which is explained by the CCR9 deficiency. When introduced via a tail vein injection and examined 20 h later by flow cytometry, RA-treated BATF KO CD4+ T cells were poorly represented in the small intestine compared with WT T cells (Fig. 6 B). The flow cytometry technique is useful for assessing cell migration into an organ but cannot accurately evaluate cell migration

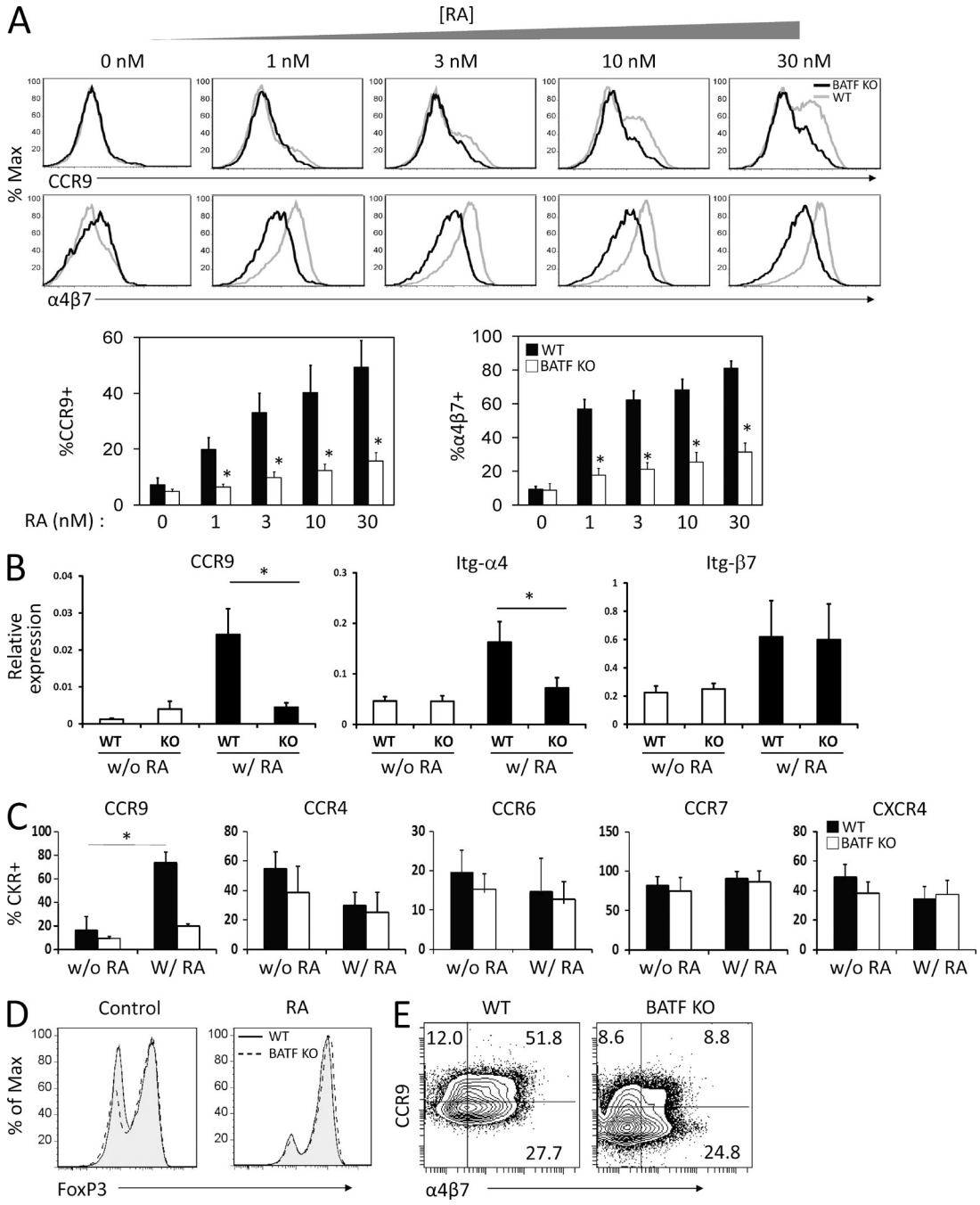

Figure 3. BATF KO T cells are defective in up-regulation of CCR9 and α4β7 but not in becoming FoxP3⁺ T cells in response to RA. (A) CD4⁺ naive T cells were activated in the presence of RA, and expression of CCR9 and α4β7 was examined by flow cytometry. (B) Expression of the trafficking receptor genes at the mRNA by cultured CD4⁺ naive T cells was examined by quantitative RT-PCR. Normalized values to β-actin levels are shown. (C) Expression of other chemokine receptors was examined by flow cytometry. (D) RA-dependent induction of FoxP3⁺ T cells in vitro from naive CD4⁺ T cells was examined by flow cytometry. Naive CD4⁺ T cells were in vitro activated with concanavalin A and IL-2 in the presence or absence of 10 nM RA for 5 d. 1 ng/ml TGFβ1 was added to culture. (E) Expression of CCR9 and α4β7 by WT and BATF KO FoxP3⁺ cells induced by RA and TGFβ1. All graphs show pooled data obtained from four experiments (*, $P < 0.05$). All error bars are SEM obtained from pooled data.

into specific microenvironments within an organ. To examine cell migration to specific tissue sites, frozen sections of the tissues were examined by confocal microscopy (Fig. 6 C). We found that migration of CD4⁺ T cells into the small and large intestinal LP and PPs was defective. Migration to MLN was decreased by 30% compared with WT T cells. One disadvantage of confocal imaging on tissue sections is that the areas viewed are limited by sectioning, and it is

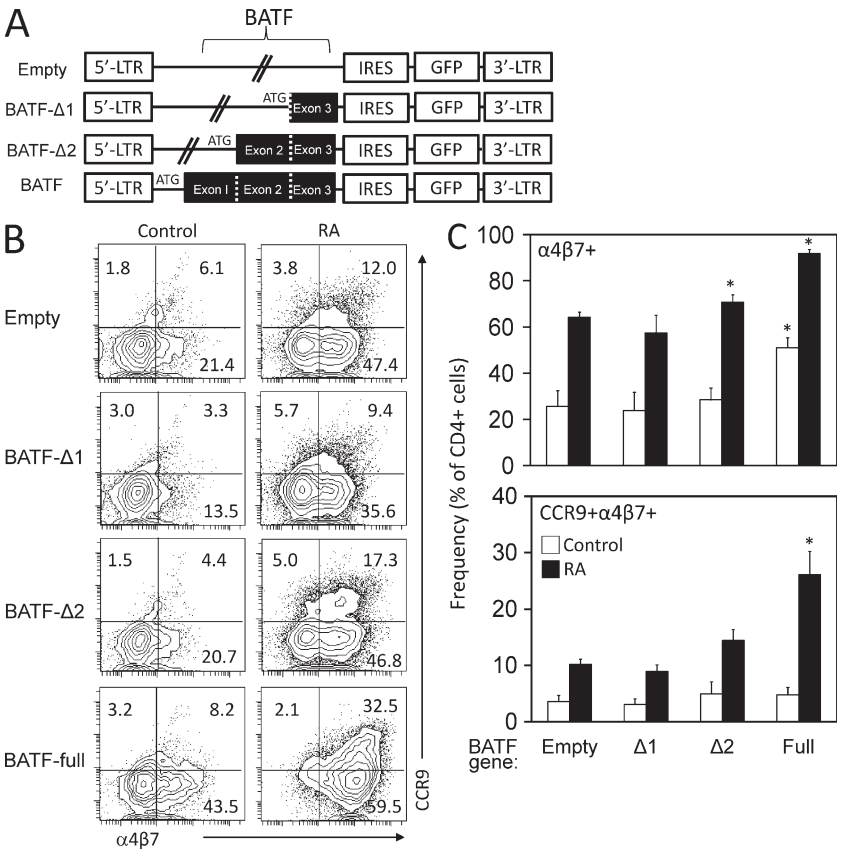

Figure 4. Enforced BATF expression restores the gut-homing receptor deficiency of BATF KO T cells. (A) Retroviral vectors expressing the full-length and truncated versions of BATF were constructed. BATF-deficient T cells were infected by retroviral vectors expressing the full-length or truncated *BATF* genes. (B and C) Expression of CCR9 and α4β7 was determined by flow cytometry 4 d after infection, and the data are shown as dot plots (B) and graphs (C). The graphs show combined data from three experiments. All error bars are SEM obtained from pooled data. Significant differences from the control groups are shown (*, $P < 0.05$).

often difficult to locate tissue sites such as small lymphoid patches in the intestine. To overcome this issue, we used multiphoton microscopy to image whole-mounted tissues (Fig. 6 D). Using this technique, we confirmed that the migration of BATF KO T cells into the small intestine and colon was severely compromised. Additionally, we found that migration of RA-conditioned BATF KO T cells to both colonic patches and PPs was defective as well. Three-dimensional (3D) images of the differential migration into the indicated whole-mounted tissues are shown in Videos 1–3.

BATF KO mice are defective in forming intestinal FoxP3$^+$ and FoxP3$^-$ T cells after oral/intragastric immunization

Oral immunization increases antigen-specific T cells, particularly FoxP3$^+$ T cells, in the intestine. We examined whether antigen-specific (OT-II) BATF KO T cells can populate the small intestine after mucosal immunization with OVA. OVA-specific BATF KO FoxP3$^+$ CD4$^+$ T cells failed to populate the small intestine (Fig. 7, A and B). However, there was no significant difference in the numbers of these cells populating other organs such as PPs, MLN, spleen, and peripheral LN (PLN). OVA-specific BATF KO FoxP3$^-$ non-T_{reg} cells also failed to populate the small intestine after immunization, whereas their population in other organs except PPs were not affected significantly (Fig. 7, A and B).

BATF KO CD4$^+$ T cells fail to induce inflammation in the intestine

BATF KO CD4$^+$ T cells may not effectively induce inflammation in the intestine because of their poor migration to the tissue. We tested this possibility using the colitis model in Rag1$^{-/-}$ mice. Rag1$^{-/-}$ mice were injected with T_{reg} cell–depleted naive CD4$^+$ T cells isolated from WT or BATF KO mice. Naive BATF KO CD4$^+$ T cells did not decrease the weight of Rag1$^{-/-}$ mice as effectively as WT T cells (Fig. 8 A). Histological examination revealed only mild inflammation in the Rag1$^{-/-}$ mice injected with naive BATF KO CD4$^+$ T cells (Fig. 8 B). This is in contrast to the severe inflammation seen in both the proximal and distal colon of the Rag1$^{-/-}$ mice injected with naive WT T cells. The numbers of Th1 cells, which are the major effector T cells to induce inflammation in this model, were decreased in the colon of Rag1$^{-/-}$ mice injected with naive BATF KO CD4$^+$ T cells (Fig. 8 C). The specific decrease of Th1 cells in the colon indicates that BATF is required for Th1 effector T cell population in the inflamed gut tissue. Also decreased were the numbers of Th17 and T_{reg} cells in the colon and the small intestine (Fig. 8 C). This decrease in Th17 cell numbers in all tissues, including the spleen, is in line with the previous reports demonstrating that BATF KO T cells are impaired in their ability to differentiate into Th17 cells (Schraml et al., 2009; Betz et al., 2010). Overall, these results demonstrate the defective population and inflammatory function of BATF KO T cells in the intestine.

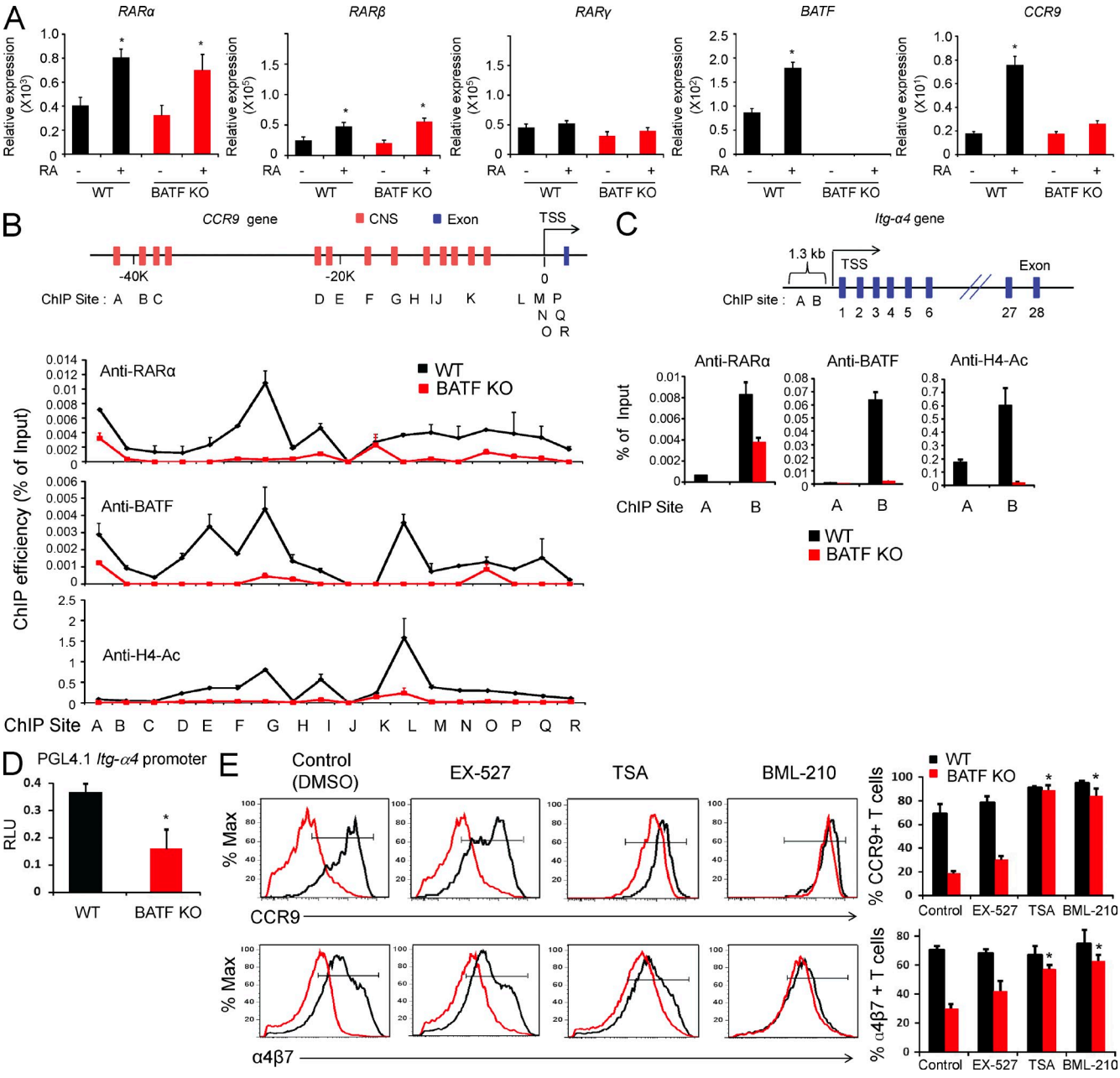

Figure 5. RARα fails to bind the 5′ regulatory regions of the mouse *CCR9* and *Itg-α4* genes in BATF deficiency. WT or BATF KO CD4+ T cells were activated for 4–5 d with concanavalin A, IL-2, and RA. (A) Expression of nuclear RAR genes in BATF KO CD4+ T cells was examined by quantitative RT-PCR. Normalized values to β-actin levels are shown. (B and C) The binding of RARα and BATF and histone H4 acetylation on the *CCR9* gene (B) or the *Itg-α4* gene (C) were assessed by ChIP assay. Representative PCR data with duplicated measurements are shown. (D) WT or BATF KO CD4+ T cells were activated for 4–5 d with anti-CD3/CD28 in the presence of IL-2 and 10 nM RA and transfected with pGL4-5′-Itg-α4. The cells were reactivated with anti-CD3/28 + IL2 in the presence of 20 nM RA for 16 h, and luciferase activity was normalized to control Renilla luciferase activity. (E) Naive WT or BATF KO CD4+ T cells were activated with anti-CD3/CD28 or concanavalin A for 4–5 d in the presence of IL-2 and 10 nM RA and the indicated HDAC inhibitors (TSA, BML-210, or EX-527), and the expression of CCR9 and α4β7 was measured by flow cytometry. Graphs show the percentage of cells expressing CCR9 or α4β7. All of the experiments were performed at least three times, and pooled (A, D, and E) or representative (B and C) data are shown. Error bars are SEM obtained from pooled data (A, D, and E) or differences between duplicated measurements (B and C). Significant differences from the KO control group are shown (*, $P < 0.05$).

The defective effector function of BATF KO CD4+ T cells may also be caused by problems in cell activation, death, or proliferation. In this regard, a lymphoproliferative disorder caused by increased T cell survival has been observed in aged mice overexpressing BATF (Logan et al., 2012). We observed that BATF KO CD4+ T cells were comparable

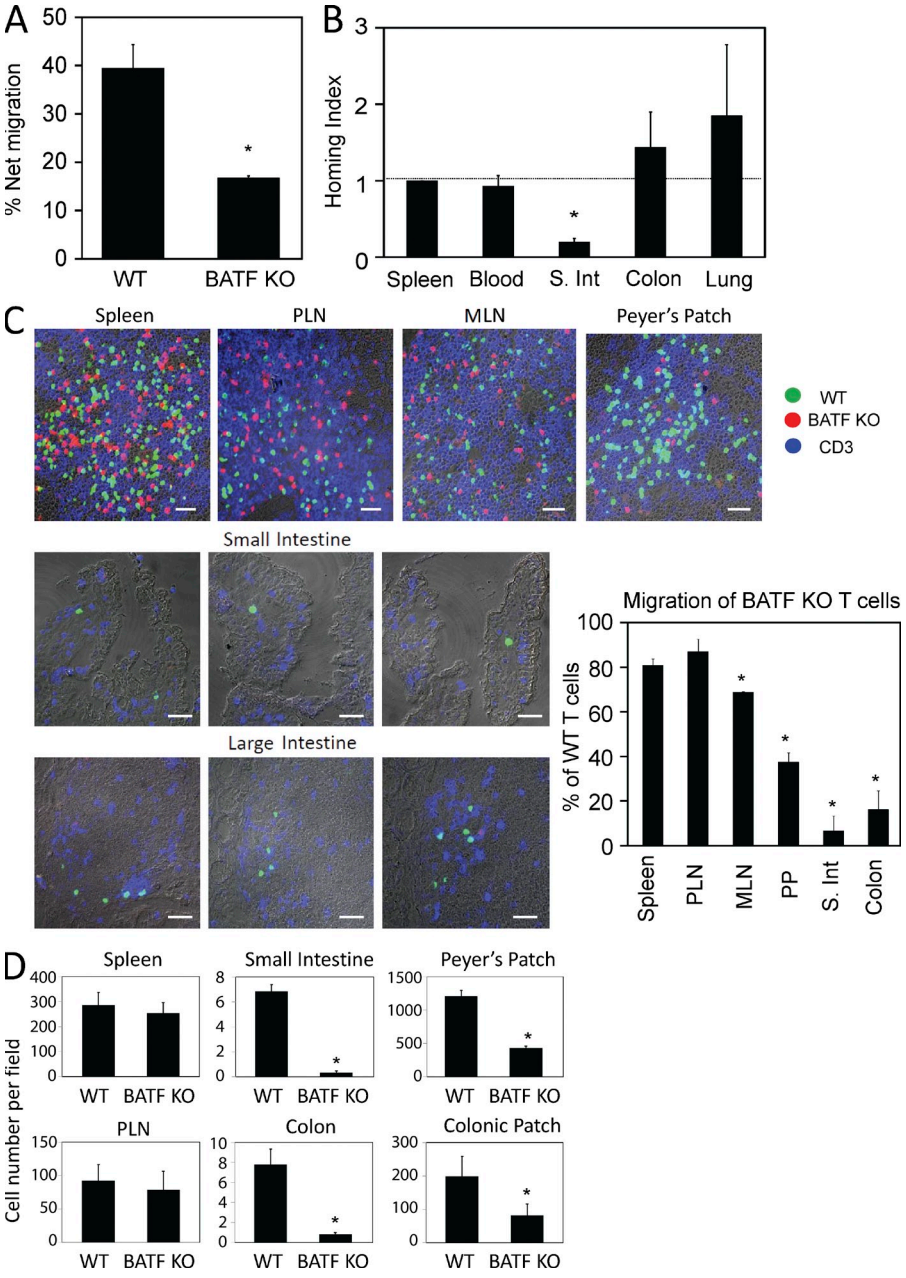

Figure 6. Defective migration of BATF KO CD4⁺ T cells into the intestine. (A) WT and BATF KO CD4⁺ T cells were activated with RA for 5 d and then added to the upper chamber in a Transwell assay. 3 µg/ml CCL25 was added to the lower chamber, and the cells in the lower chamber were counted after 3 h. The data are shown as percent net migration after normalization to input cells and subtraction of the background migration rates. (B) WT and BATF KO CD4⁺ T cells were activated for 5–6 d with concanavalin A, IL-2, and RA and were then differentially labeled with CFSE and TRITC. The cells were mixed in a 1:1 ratio and injected i.v. into WT mice. (B and C) Numbers of cells in the indicated organs were assessed by flow cytometry (B) or confocal microscopy (C) after 20 h. Bars, 50 µm. (B) The homing index indicates the frequency of KO cells relative to WT cells. (D) The tissues were examined also with multiphoton microscopy. Pooled data from three experiments are shown in A and B. Pooled data from multiple images (3–12) obtained from two independent experiments are shown in C and D. All error bars are SEM obtained from pooled data. Significant differences between WT and BATF KO T cells are shown (*, $P < 0.05$).

with WT T cells in loss of CD62L and up-regulation of CD69 in response to anti-CD3/28 + IL-2 (Fig. 9 A). Moreover, BATF KO CD4⁺ T cells did not display significant differences in cell death after T cell activation (Fig. 9 B). Expansion of OT-II CD4⁺ T cells after parenteral immunization was also largely intact (Fig. 9 C). Similarly, homeostatic CD4⁺ T cell expansion in lymphopenic Rag1$^{-/-}$ mice was slightly decreased but was not defective (Fig. 9 D). Absolute numbers of BATF KO CD4⁺ T cells in the spleen and PLN were comparable with WT, but the numbers in the intestinal LP and the intestine-draining LN (MLN) were decreased as expected (Fig. 9 E). These results indicate that general cell processes such as cell activation, death, and proliferation appear largely intact in BATF KO T cells.

BATF KO iT$_{reg}$ cells fail to suppress inflammation in the intestine

As shown in Fig. 3 E, iT$_{reg}$ cells derived from naive BATF KO CD4⁺ T cells were deficient in the expression of CCR9 and α4β7. We examined whether this deficiency would affect the in vivo suppressive function of BATF KO iT$_{reg}$ cells. RA-treated BATF KO iT$_{reg}$ cells were as effective as WT iT$_{reg}$ cells in suppressing responder T cells in vitro (Fig. 10 A). Thus, function of the BATF KO iT$_{reg}$ cells is intact in vitro. We next compared the suppressive functions of CD45.2⁺ iT$_{reg}$ cells generated from naive WT and BATF KO CD4⁺ T cells in vivo in the T cell–induced colitis model. RA-treated BATF KO iT$_{reg}$ cells were not as effective as WT T$_{reg}$ cells in suppressing the weight loss induced by the inflammatory

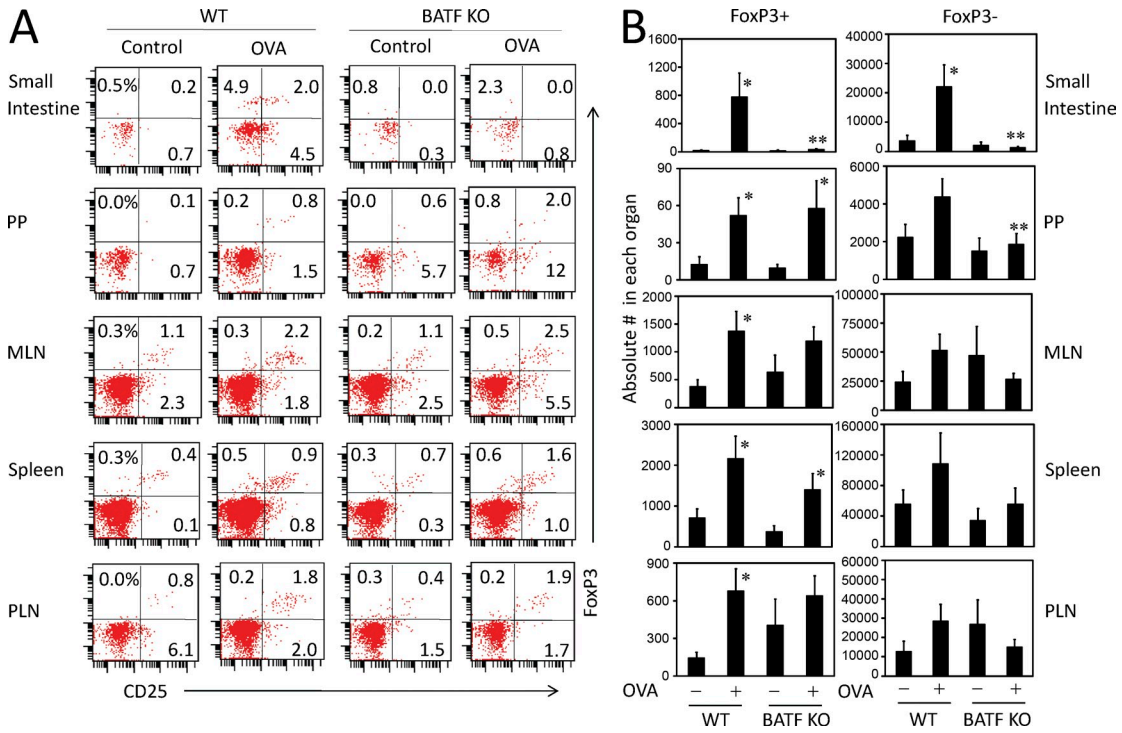

Figure 7. Defective population of CD4⁺ T cells in the intestine after intragastric immunization. WT and BATF KO OT-II CD4⁺ T cells were transferred into CD45.1⁺ mice, and the mice were intragastrically immunized with OVA. The mice were sacrificed 12 d later. (A) CD4⁺ CD45.2⁺ T cell subsets in the intestine and other organs were examined by flow cytometry. (B) Absolute numbers of CD4⁺ CD45.2⁺ FoxP3⁺ and FoxP3⁻ CD4⁺ T cells in various organs are shown. Pooled data obtained from three experiments (n = 5–7/group) are shown. All error bars are SEM obtained from pooled data. Significant differences ($P < 0.05$) from non-OVA–treated groups (*) or WT counterparts (**) are shown.

activity of WT T cells (Fig. 10 B). Histological examination revealed that only WT iT_{reg} cells, but not BATF KO iT_{reg} cells, effectively suppressed colitis (Fig. 10 C). BATF KO iT_{reg} cells were also less effective than WT iT_{reg} cells in decreasing the number of CD45.1⁺ (WT naive T cell derived) Th1 effector cells in the MLN and colon (Fig. 10 D). Th17 cells, which are generally suppressed by Th1 cells, were reciprocally increased with injection of WT iT_{reg} cells but not with BATF KO iT_{reg} cells. Fewer CD45.2⁺ BATF KO iT_{reg} cells than WT iT_{reg} cells were found in the colon but not in the MLN of the $Rag1^{-/-}$ mice (Fig. 10 E), indicating defective population of the transferred BATF KO iT_{reg} cells in the colon but not in the MLN in the inflammatory condition. Overall, these results demonstrate that BATF is required for the in vivo population of T_{reg} cells in the gut and their regulatory activity on the Th1 cell activity and tissue inflammation.

DISCUSSION

Our study identified BATF as a cellular factor required for the optimal expression of CCR9 and α4β7 by T cells, particularly CD4⁺ T cells, in response to RA. We provided evidence that naive CD4⁺ T cells require BATF to up-regulate the gut-homing receptors during T cell activation and differentiation. BATF is important for the expression of gut-homing receptors by both effector cells and FoxP3⁺ T_{reg} cells. Moreover, this role of BATF in regulating gut-homing receptor expression is important not only for effector T cells in inducing intestinal inflammation but also for T_{reg} cells in controlling inflammation. The findings implicate BATF as a key regulator of T cell–mediated immune responses and inflammation in the intestine.

Our results indicate that BATF deficiency makes CD4⁺ T cells less responsive to the RA signal in up-regulating the gut-homing receptors. RA, produced by gut epithelial cells and dendritic cells, is the major inducer of CCR9 and α4β7. In this process, RARα plays an important role as the receptor for RA and works together with the TCR activation signal to up-regulate CCR9 (Iwata et al., 2004). RA also plays an important role in up-regulating the Itg-α4 subunit of the α4β7 integrin (Kang et al., 2011). The Itg-β7 subunit of α4β7 is expressed constitutively and increased further by TGFβ1 (Kang et al., 2011). Our results indicate that expression of *Itg-α4*, but not *Itg-β7*, is defective at the mRNA level in BATF deficiency.

Defective expression of the gut-homing receptors by BATF KO CD4⁺ T cells can result in inefficient migration of these T cells into the intestine. The CCR9 ligand, CCL25 (also called TECK), is highly expressed in the epithelial cells of the small intestine (Kunkel et al., 2000). CCR9 deficiency leads to defective homing of memory T cells into the small intestinal LP (Stenstad et al., 2006). α4β7 is required for migration into the whole intestine because its adhesion receptor, MAdCAM-1, is expressed on intestinal endothelial cells (Sigmundsdottir and Butcher, 2008). MAdCAM-1 is expressed also on the

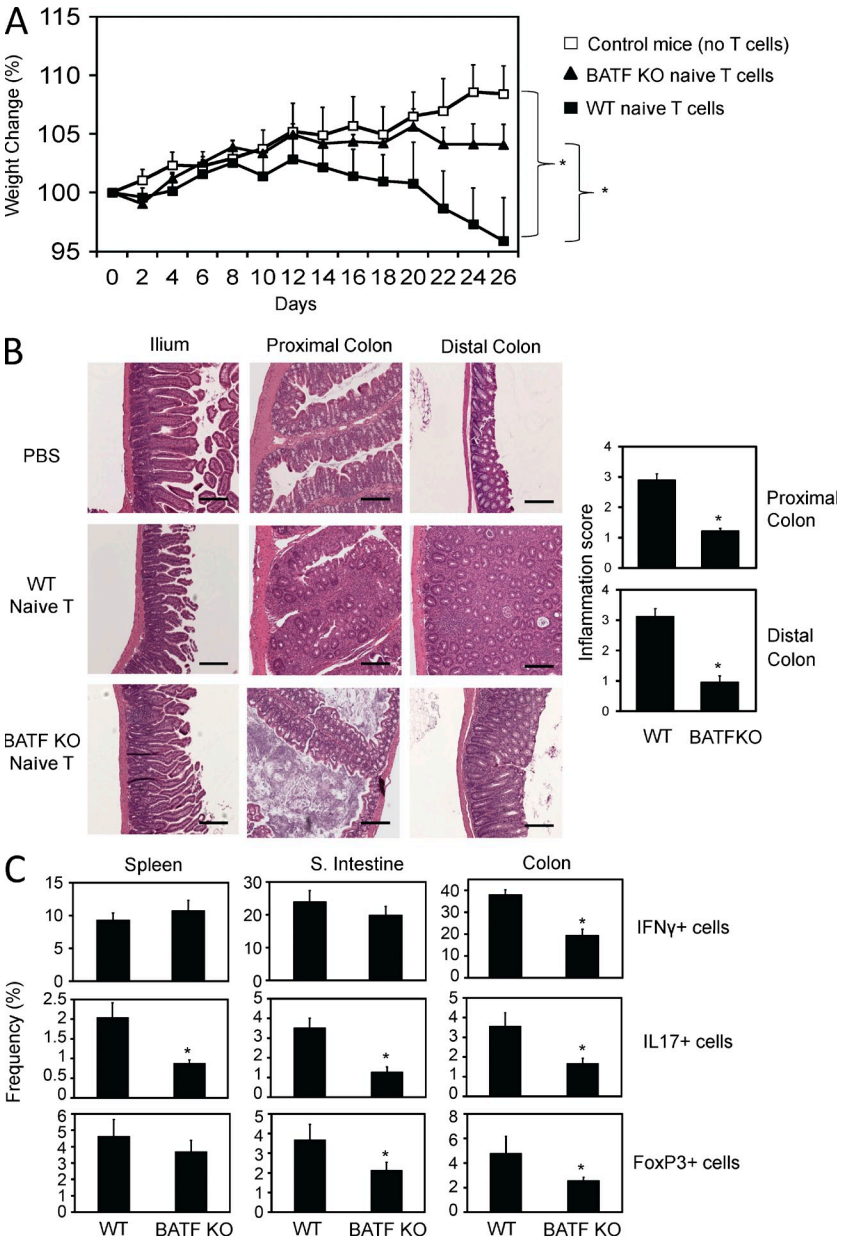

Figure 8. BATF KO T cells are defective in inducing colitis. Rag1$^{-/-}$ mice were injected with the naive CD4$^+$CD25$^-$ T cells isolated from WT or BATF KO mice. (A) Weight change after injection of WT or BATF KO naive CD4$^+$CD25$^-$ T cells was monitored. (B) Histological changes of the intestine of Rag1$^{-/-}$ mice injected with naive CD4$^+$CD25$^-$ T cells were examined at the time of mouse termination. Bars, 200 µm. (C) Frequencies of Th1, Th17, and FoxP3$^+$ T cells in Rag1$^{-/-}$ mice injected with naive CD4$^+$CD25$^-$ T cells were determined by flow cytometry. Pooled data obtained from three experiments are shown in the graphs ($n \geq 10$/group). All error bars are SEM obtained from pooled data. Significant differences between indicated groups or from WT groups are shown (*, $P < 0.05$).

endothelial cells in PPs (Streeter et al., 1988), and therefore, BATF KO T cells with low α4β7 expression are inefficient for migration into PPs. Additionally, migration of BATF KO T cells to colonic patches was defective, indicating that BATF expression is important for T cell migration into the gut-associated lymphoid tissues and nonlymphoid tissue areas.

Oral or intragastric immunization induces immune tolerance in the intestine in part by generating gut-homing T_{reg} cells in the intestine (Thorstenson and Khoruts, 2001; Nagatani et al., 2004). Gut-homing receptors such as CCR9 and α4β7 are required for the proper induction of immune tolerance in the intestine, probably because of the need for efficient migration of T_{reg} cells into the intestine in the process (Cassani et al., 2011). Our results revealed that BATF deficiency leads to defective population of T_{reg} cells in the small intestine after oral immunization. Also defective was the population of non-T_{reg} cells in the intestine. Although the short-term homing of T cells into PPs is somewhat defective, oral immunization still normally induced population of T cells in PPs and other lymphoid tissues such as spleen and PLN. This is perhaps because T cell migration to secondary lymphoid tissues involves trafficking receptors that are not regulated by BATF. In this regard, it has been shown that T cell migration into PPs can be mediated also by an α4β7-independent mechanism mediated by L-selectin (Bargatze et al., 1995; Warnock et al., 2000).

Our results indicate that BATF deficiency has a significant functional consequence on the induction and regulation of tissue inflammation in the intestine. BATF KO T cells fail to induce colitis in Rag1$^{-/-}$ mice. Also, BATF KO FoxP3$^+$ T cells fail to suppress colitis induced by inflammatory T cells.

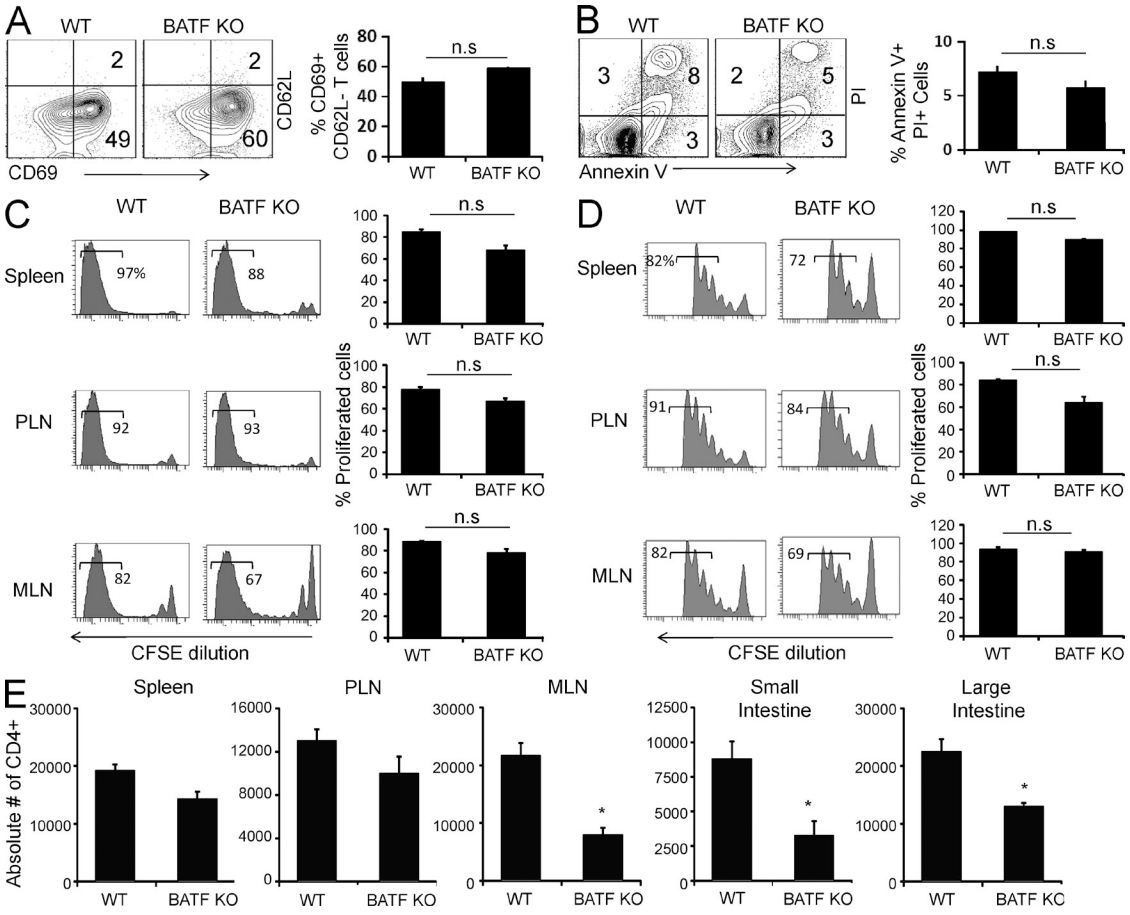

Figure 9. BATF KO T cells are largely intact in activation, survival, and proliferation. (A–E) WT and BATF KO CD4$^+$ T cells were examined in vitro for activation (A) and survival (B) and in vivo for proliferation (C–E). T cells were activated with anti-CD3, anti-CD28, and IL-2 for 4 d, and expression of CD69 and CD62L (A) and cell death based on staining with annexin V and propidium iodide (PI; B) were examined. (C) CD45.1 mice were injected i.v. with 10 million CFSE-labeled WT or BATF KO OT-II CD4$^+$ T cells and were immunized i.p. with OVA in complete Freund's adjuvant. CFSE dilution was examined by flow cytometry 5 d later. (D) Rag1$^{-/-}$ mice were injected i.v. with 10 million CFSE-labeled WT or BATF KO CD4$^+$ T cells. The host mice were examined 14 d later for CFSE dilution. (E) Absolute numbers of WT and BATF KO CD4$^+$ T cells expanded in the indicated organs of Rag1$^{-/-}$ mice are shown. Pooled data obtained from three experiments are shown in the graphs ($n \geq 10$/group). All error bars are SEM obtained from pooled data. Significant differences from WT groups are shown (*, $P < 0.05$).

In an inflammatory condition, BATF KO effector Th1 cells and FoxP3$^+$ T cells failed to populate the colon. These results suggest that BATF is important for the generation of functionally competent effector and suppressor T cells with a normal gut migratory capacity. Although this study focuses on the migration aspect, BATF is known to have additional functions in regulation of T cell function. Although BATF is not required to generate Th1 cells and FoxP3$^+$ T cells, BATF is required to generate Th17 cells and T-Fh cells. It has been reported that BATF is required for generation of effector CD8$^+$ T cells (Kuroda et al., 2011) and for a check point in the self-renewal of hematopoietic stem cells in response to DNA damage (Wang et al., 2012). Thus, other phenotypes of BATF KO mice in addition to the migration defect could contribute to the ineffective function of effector and suppressor T cells in control of tissue inflammation in the intestine. Nevertheless, we did confirm that the basic activation, survival, and expansion of BATF KO T cells in response to antigens and in a lymphopenic condition are not impaired.

The result of our retroviral gene transfer study demonstrated that BATF expression allows BATF KO T cells to regain sensitivity to the RA signal and up-regulate the two homing receptors. How BATF regulates gene expression and cell function is an active area of research. The in vitro analysis of BATF function indicates a role for the protein in the suppression of AP-1 transcriptional activity (Echlin et al., 2000). BATF inhibits expression of the nicotinamide adenine dinucleotide (NAD$^+$)–dependent deacetylase Sirt1, which in turn results in increased T-bet expression (Kuroda et al., 2011). In contrast, BATF has been shown to be associated with transcriptional activation of several genes in Th17 cells and with the induction of AID (Schraml et al., 2009; Ise et al., 2011). BATF and IRF-4 can cooperate to induce the *IL10*, *IL-17a*, and *IL-21* genes in T cells and to regulate the development

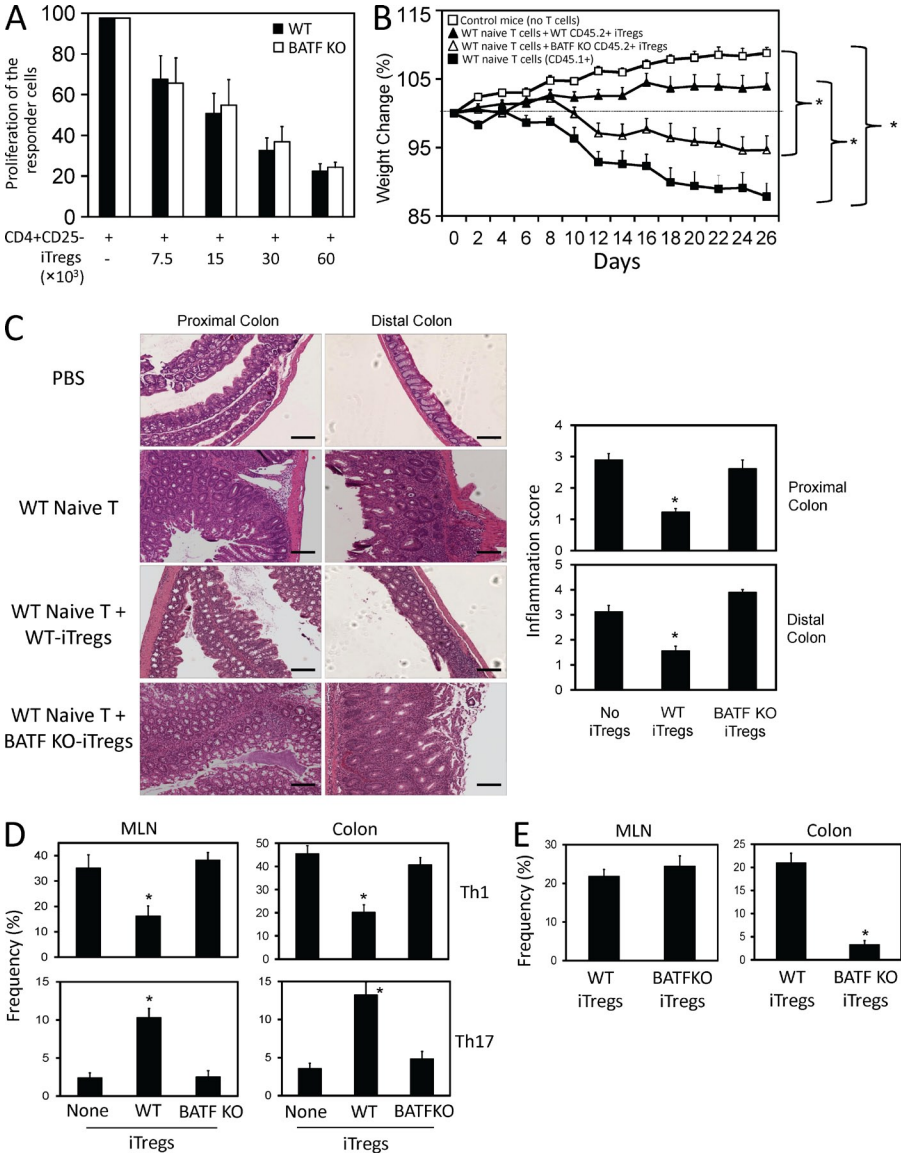

Figure 10. BATF KO iT_{reg} cells, although equally suppressive in vitro as WT iT_{reg} cells, are unable to suppress colitis. BATF KO and WT iT_{reg} cells were prepared in vitro from naive $CD4^+$ T cells through activation with concanavalin A in the presence of TGFβ1 and 10 nM RA for 6 d. (A) The iT_{reg} cells were examined for their suppressive activity on proliferation of target ($CD4^+CD25^-$) T cells based on frequencies (percentages) of CFSE-diluted cells determined by flow cytometry. (B–E) $Rag1^{-/-}$ mice were injected i.p. with 0.6×10^6 WT $CD45.1^+$ naive $CD4^+CD25^-$ T cells and 1.2×10^6 WT or BATF KO $CD45.2^+$ iT_{reg} cells, and weight change (B), histological changes on day 25–28 (C), frequencies of Th1, Th17, and $FoxP3^+$ T cells derived from the naive $CD4^+CD25^-$ T cells (D), and frequencies of $FoxP3^+$ cells derived from the injected iT_{reg} cells (E) were examined. Bars, 200 µm. The mice (n = 9–10/group) were sacrificed on day 25–28 after T cell transfer when some mice lost >20% of their original weight. Pooled data obtained from three experiments are shown in the graphs. All error bars are SEM obtained from pooled data. Significant differences between indicated groups or between WT and BATF KO groups are shown (*, $P < 0.05$).

of $CD8\alpha^+$ dendritic cells (Glasmacher et al., 2012; Li et al., 2012; Tussiwand et al., 2012). Several BATF- and RARα-binding sites are present in the 5′ regulatory regions of the *CCR9* and *Itg-α4* genes. With one exception, all of these binding sites on the *CCR9* gene and all of the BATF-binding sites on the *Itg-α4* gene do not possess the features of AP-1–IRF composite elements, suggesting that the expression of the gut-trafficking receptors would likely involve a different regulatory mechanism. Histone modification by acetylation is critical for chromatin remodeling for gene expression in response to nuclear hormone receptor ligands (Rosenfeld et al., 2006). Our results indicate that RAR binding and histone acetylation at the *CCR9* and *Itgα4* genes are important for expression of the gut-homing receptors and are regulated by BATF. The detailed interaction underlying the regulation of the trafficking receptor genes will require further study as it may involve cross talk between BATF and the RAR expression machinery and the activities of several coactivators and repressors. In conclusion, this study identified a novel function for BATF in up-regulating the major gut-homing receptors on T helper cells.

MATERIALS AND METHODS

Mice. Batf ΔZ/ΔZ mice with the third exon deleted in the *Batf* gene have been described previously (Betz et al., 2010) and will be referred to as BATF KO mice in this article. C57BL/6 mice breeding pairs were purchased from Harlan. BATF KO mice were backcrossed on the C57BL/6 background for >10 generations. Congenic CD45.1 C57BL/6, OT-II (C57BL/6-Tg(TcraTcrb)425Cbn/J), and $Rag1^{-/-}$ mice (B6.129S7-Rag1tm1Mom/J) were obtained from the Jackson Laboratory. OT-II mice were crossed to BATF KO mice to generate OT-II BATF KO mice. All mice used in this study were maintained at Purdue University, and experiments were performed according to approved protocols by the Purdue University Animal Care and Use Committee. Generally, the mice were used between 6 and 8 wk of age at the start of each experiment.

Cell culture. Single cell suspensions of LNs (mesenteric, inguinal, auxiliary, and brachial) and spleens were prepared by grinding tissues through an iron mesh. $CD4^+$ T cells were isolated by a $CD4^+$ T cell isolation kit (Miltenyi Biotec). The cells were further processed for isolation of T_{reg} cell–depleted $CD4^+CD25^-CD44^-CD69^-$ naive T cells with an AutoMACS separator (Miltenyi Biotec) as previously described (Kang et al., 2011). The cells were cultured for 5–6 d in complete RPMI 1640 medium (10% FBS) supplemented with 100 U/ml hIL-2. When indicated, 1 ng/ml hTGF-β1 and/or 10 nM RA (all-trans RA) was added. As T cell activators, 2.5 µg/ml concanavalin A or $OVA_{323–339}$ peptide (at 1 µg/ml with 3× irradiated splenocytes for OT-II cells) was used. All cytokines were obtained from R&D Systems or PeproTech. When indicated, HDAC inhibitors such as 10 µM EX-527 (Sigma-Aldrich), 5 nM trichostatin (Enzo Life Sciences), and 10 µM BML-210 (Sigma-Aldrich) were added to the T cell culture.

Flow cytometry. Expression of chemokine receptors and integrins was examined as previously described (Kang et al., 2011). Single cell suspensions, isolated from various organs of mice, were stained with antibodies to CCR9 (clone 242503) and α4β7 (clone DATK32). For cells stained with unconjugated antibodies, biotin-labeled secondary antibodies and tertiary PE/PerCP/APC-streptavidin (BD) were used along with antibodies to CD44 and CD4. When necessary, the cells were further stained with antibodies to FoxP3 (FJK-16s; eBioscience) according to the manufacturer's protocol.

Chemotaxis assay. The chemotaxis assay was performed and analyzed as described previously (Wang et al., 2010). In brief, 5×10^5 T cells, activated in the presence or absence of 10 nM RA for 5 d, were added to the upper chamber of Transwell inserts (Corning) and allowed to migrate to the lower chamber containing 3 µg/ml murine CCL25 (R&D Systems) for 3 h. The cells in the lower chambers were collected, stained, and quantitated by flow cytometry.

Retroviral expression of BATF. The full-length and truncated coding regions of the mouse *BATF* cDNA were amplified by PCR using the primers described in Table S2. Amplified DNA fragments were cloned into a retroviral vector with GFP expression to help identify transduced cells (Wang et al., 2009) and sequenced for accuracy. A retrovirus packaging cell line (Phoenix-ECO) was transfected with the vectors, and activated T cells were infected with culture supernatant containing the recombinant virus. The T cells were previously activated for 36 h with IL-2 and concanavalin A in the presence or absence of 10 nM RA. The virus-infected T cells were harvested 4 d later, and GFP^+ T cells were examined for CCR9 and α4β7 expression.

Bioinformatics and ChIP assays. The VISTA bioinformatics tool was used to determine conserved noncoding sequences. ChIP assays were performed as described previously (Kang et al., 2011). $CD4^+$ T cells (5×10^6 naive per sample), cultured in the presence of 100 U/ml IL-2, 1.5 µg/ml concanavalin A, and 10 nM RA for 4–5 d, were used for ChIP assays. DNA protein complexes were immunoprecipitated using 4 µg anti-BATF (rabbit polyclonal; Cell Signaling Technology), anti-RARα (rabbit polyclonal; Santa Cruz Biotechnology, Inc.), and anti–acetyl-histone H4 antibodies (EMD Millipore). Real-time PCR detection using the primers described in Table S3 was conducted with a 7500 Sequence Detection System using the SYBR green Master Mix (Applied Biosystems).

Transfection and reporter assay. Before transfection, T cells were cultured with 10 nM RA and 100 U/ml IL-2 with plate-bound 5 µg/ml anti-CD3 and 2 µg/ml anti-CD28 for 4 d in complete RPMI medium replaced every 2 d. Activated $CD4^+$ T cells (5×10^6 cells per sample) were transfected with 20 µg pGL4.1-5′-Itg-α4 (pGL4.1 harboring the 1.3 kbp 5′ regulatory region of the mouse *Itg-α4* gene) and 3.5 µg pRL with the Mouse T Cell Nucleofector kit (Lonza). The cells were rested at 37°C for 4–5 h and activated with plate-bound anti-CD3, anti-CD28, and 100 U/ml IL-2 in the presence 100 nM RA for 16 h before measurement with the Dual Luciferase Assay System (Promega). Relative light units (RLU) after normalization of firefly luciferase activity with Renilla luciferase (pRL) activity are shown.

Short-term homing experiments and confocal analysis to determine localization of injected cells. For homing experiments, naive $CD4^+$ T cells isolated from splenocytes and LN cells of WT and BATF KO mice were cultured for 5–6 d in complete RPMI 1640 medium (10% FBS) supplemented with 2.5 µg/ml concanavalin A, 25 U/ml hIL-2, and 10 nM RA. WT cells (10^7 cells/mouse; labeled with CFSE; Invitrogen) and BATF KO cells (10^7 cells/mouse; labeled with tetramethylrhodamine isothiocyanate; Invitrogen) were coinjected i.v. into normal C57BL/6 mice. The host mice were sacrificed ∼20 h later, and indicated organs were harvested. The numbers of injected $CD4^+$ cells migrated into each organ were determined with flow cytometry. Absolute numbers of T cells that migrated into various organs and homing index were determined as described previously (Wang et al., 2010).

Confocal and multiphoton microscopy. Spleen, MLN, small intestine, colon, and PPs were harvested from 8–10-wk-old mice and frozen in Tissue-Tek OCT Compound (Sakura). The tissue blocks were cut into 6-µm sections, fixed in cold acetone, and stained with combinations of FITC/PE/APC-conjugated antibodies to CD4 (RM4-5), cytokeratin (AE1/AE3), and CD8 (53–6.7). The images were collected with an LSM 710 (Carl Zeiss) or SP5 II (Leica) confocal microscope. The 3D images were acquired on paraformaldehyde-fixed whole-mounted tissues with an SP5 multiphoton System (Leica) with Mai Tai Deep See Tunable IR Laser (IR laser set at 800 nm; filters were 430–480 for second harmonic generation, 500–595 for CFSE, and 595–605 for TRITC). Acquisition volume was ∼300 × 300 × 100 µm, and z-axis resolution was 0.5 µm.

T cell population in the intestine after intragastric immunization. T_{reg} cell–depleted $CD4^+CD25^-$ T cells were isolated from WT OT-II and BATF KO OT-II (CD45.2) mice. The OT-II T cells (10^7 cells/mouse) were adoptively transferred i.v. into CD45.1 congenic mice. 2 d later, the recipient mice were injected with OVA protein (100 mg/injection in 200 µl of PBS; Sigma-Aldrich) intragastrically with a round-tip needle. On the next day, this immunization was repeated once. 12 d later, mice were sacrificed to examine the numbers of $CD4^+$ $FoxP3^+$ and $FoxP3^-$ T cells in indicated organs.

In vitro T_{reg} cell assay. For assessing the T_{reg} cell activity, CFSE-labeled $CD4^+CD25^-$ T cells (target cells, 3×10^4 cells/well) and iT_{reg} cells as suppressors were co-cultured in round-bottom 96-well plates for 3 d at the indicated ratios with 2 µg/ml anti-CD3 antibody and irradiated splenic cells (9×10^4 cells/well). iT_{reg} cells were generated in vitro with culturing $CD4^+CD25^-$ naive T cells for 6 d in the presence of 1 ng/ml TGFβ1, 10 nM RA, and 2.5 µg/ml concanavalin A. Dilution of CFSE indicating cell proliferation was determined by flow cytometry.

Induction of colitis in $Rag1^{-/-}$ mice and in vivo assessment of T_{reg} cell function. For assessment of effector T cell function, $Rag1^{-/-}$ mice were injected i.p. with 0.6×10^6 WT or BATF KO naive T cells. For comparison of T_{reg} cell function, 0.6×10^6 WT naive T cells were injected together with 1.2×10^6 WT or BATF KO iT_{reg} cells. Weight change was monitored, and the mice were sacrificed on day 25–28 after T cell transfer when some mice lost >20% of their original weight. Intestinal inflammation in $Rag1^{-/-}$ mice was scored as previously described (Wang et al., 2010) based on the degree of leukocyte infiltration, mucosal hyperplasia, and loss of villi on a scale of 0–4. The histological images were obtained with a wide-field DM2000 microscope equipped with a DFC295 color camera (Leica) at 100 magnification.

T cell proliferation in vivo. For assessment of T cell proliferation in vivo, 10^7 CFSE-labeled $CD4^+$ T cells were injected i.v. into $Rag1^{-/-}$ mice, and the host mice were sacrificed on day 14. For assessment of antigen-dependent T cell proliferation, CD45.1 congenic mice were injected i.v. with 10^7 CFSE-labeled WT or BATF KO OT-II $CD4^+$ T cells and immunized i.p. with 250 µg OVA in complete Freund's adjuvant. The host mice were sacrificed 5 d

later. CFSE dilution and absolute numbers of donor $CD4^+$ T cells in indicated tissues were examined.

Statistical analyses. Means with SEM are shown in most of the figures. Student's paired *t* test (two tailed) was used to determine the significance of differences between two groups. The weight change data in Figs. 8 and 10 was examined with a repeated measures ANOVA (SAS version 9.2; SAS Institute Inc.). P-values ≤ 0.05 were considered significant.

Online supplemental material. Fig. S1 shows the regulatory regions of *CCR9* and *Itg-α4* genes and the binding sites for RARα and BATF examined in this study. Videos 1–3 show 3D images of the WT and BATF KO T cells migrated into the spleen and PLN (Video 1), a PP and colonic patch (Video 2), and the small intestinal LP and colon (Video 3). Tables S1–S3 list primer sequences and putative binding sites for RAR and BATF. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20121088/DC1.

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